# Method of producing double low restorer lines of Brassica napus having a good agronomic value

The invention relates to a method of producing a double low restorer lines of Brassica napus for Ogura cytoplasmic male sterility (cms) presenting a radish introgression carrying the Rfo restorer genes deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterized by female fertility, a good transmission rate of Rfo and a high vegetative vigour. The invention relates also to a method of forming Brassica napus hybrid seed and progeny thereof and to the use of markers for selection.

Breeding restorer lines for the Ogu-INRA Cytoplasmic Male Sterility (cms) system in rapeseed (Brassica napus L.) has been a major objective during the past few years. Extensive backcross and pedigree breeding were necessary to improve their female fertility and to get double low restorer lines. The so-called « double low » varieties are those low in erucic acid in the oil and low in glucosinolates in the solid meal remaining after oil extraction. However some difficulties can still be encountered in breeding these lines (introgression rearrangements, possible linkage with negative traits) due to the large size of the radish introgression.

The inventors thus assigned themselves the objective of providing a new improved double low restorer line with a good agronomic value.

This objective is obtained by a new method of producing a recombined double low restorer line for the Ogu-INRA cms in rapeseed.

A first object of the present invention relates to a method of producing double low restorer lines of Brassica napus for Ogura cytoplasmic male sterility (cms) presenting radis introgression carrying the Rfo restorer gene deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour, said method including the step of:

a) crossing double low cms lines of spring Brassica napus comprising a deleted radish insertion with the double low line of spring Drakkar for forming heterozygous restored plants of Brassica napus,

b) irradiating before meiosis the heterozygous restored plants obtained in step a) with gamma ray irradiation,

c) crossing pollen from flowers obtained in step b) with the cms double low spring Wesroona line,

d) testing the progeny for vigour, female fertility and transmission rate of the cms gene,

e) selecting progeny lines.

In the present invention, the term "lines(s)" means a plant which is essentially homozygote and which is reproducible by auto-pollination.

A method according to claim 1, wherein the irradiation dose in step b) is 65 Gray during 6 mn.

According to one advantageous form of embodiment of the method according to the present invention, the double low cms line of spring Brassica napus of step a) is R211.

R211 is an INRA spring restorer line.

Drakkar is a French spring registered variety.

Wesroona is an Australian spring registered variety.

According to one advantageous form of embodiment of the method according to the present invention, the testing is performed with the combination of five markers selected from PGIol, PGIUNT, PGIint, BolJon and CP418.

Another object of the present invention relates to double low restorer lines of Brassica napus for Ogura cms presenting a Rfo insertion deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour.

According to one advantageous form of embodiment, the double low restorer lines present a unique combination of five markers selected from PGIol, PGIUNT, PGIint, BolJon and CP418.

Another object of the present invention relates to a method of forming Brassica napus hybrid plants and progeny thereof obtained though the steps of:

a) providing a restorer line produced according to claim 1 and bred to be homozygous,

b) using said restorer line in a hybrid production field as the pollinator,

c) using cms sterile plants in a hybrid production field as the hybrid seed producing plant, and

d) harvesting the hybrid seed from the male sterile plant.

Another object of the present invention relates to seeds of Brassica plant obtained from the methods according to the present invention.

Still another object of the invention relates to seeds of Brassica napus deposited in NCIMB Limited, 23 St Machar Drive, Aberdeen, Scotland, AB24 3RY, UK, on July 4 , 2003, under the reference number NCIMB41183.

Another object of the present invention relates to the use of at least four markers PGIol, PGIint, BolJon and CP418, or any portion of them comprising at least one polymorphic site, for characterising recombined restorer lines of Brassica napus for Ogura cms presenting a Rfo insertion deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour.

In a preferred embodiment, the combination is of five markers PGIol, PGIUNT, PGIint, BolJon and CP418.

In the present invention, the expression " any portion of them comprising at least one polymorphic site" means any part of the sequence showing at least a difference between the B.oleracea type sequence and B.rapa type sequence.

Such markers are represented in the following figures and sequence listing for the R2000 line.

According to one advantageous form of embodiment, the present invention relates to:

- The marker PGIol which is amplified using the primers: PGIol U and PGIol L
  (PGIol U: 5'TCATTTGATTGTTGCGCCTG3';
  PGIol L: 5'TGTACATCAGACCCGGTAGAAAA3')
- The marker PGIint which is amplified using the primers: PGIint U and PGIint L
  (PGIint U: 5'CAGCACTAATCTTGCGGTATG3';
  PGIint L: 5'CAATAACCCTAAAAGCACCTG3')
- The marker PGIUNT which is amplified using the primers: PGIol U and PGIint L:

(PGIol U: 5'TCATTTGATTGTTGCGCCTG3';

PGIint L: 5'CAATAACCCTAAAAGCACCTG3')

- The marker BolJon which is amplified using the primers: BolJon U and BolJon L:

(BolJon U: 5'GATCCGATTCTTCTCCTGTTG3';

BolJon L: 5'GCCTACTCCTCAAATCACTCT3')

- The marker CP418 which is amplified using the primers: SG129 U and pCP418 L:

(SG129 U: cf Giancola et al, 2003 *Theor Appl. Genet. (in press)*

pCP418 L : 5'AATTTCTCCATCACAAGGACC3')

Another object of the present invention relates to the PGIol, PGIUNT, PGIint, BolJon and CP418 markers whose sequences follow:

PGIol R2000 marker:

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TCATTTGATT GTTGCGCCTG TCGCCTTGTT GTGTTATGAT GAATGAACAG CAGTCATTTA  60
ACATGTGGTT AACTTAACAG GGCTCCGGCT GTTGCAAAAC ACATGGTTGC TGTCAGCACT 120
AATCTTGCGG TATGAATTTG TGATTAAATT TGTTTGTTTG TGACTCTTTC TTCATTGTTC 180
GTTTTCGTAC AATAAACCGA ATGTATAATC TTTTTACAAA CTGAATTTTC TACCGGGTCT 240
GATGTACA                                                          248
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PGIUNT R2000 marker:

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TCATTTGATT GTTGCGCCTG TCGCCTTGTT GTGTTATGAT GAATGAACAG CAGTCATTTA  60
ACATGTGGTT AACTTAACAG GGCTCCGGCT GTTGCAAAAC ACATGGTTGC TGTCAGCACT 120
AATCTTGCGG TATGAATTTG TGATTAAATT TGTTTGTTTG TGACTCTTTC TTCATTGTTC 180
GTTTTCGTAC AATAAACCGA ATGTATAATC TTTTACAAAC TGAATTTTCT ACCGGGTCTG 240
ATGTACAATG CTAGTCTCCA TGTTCTTGGG GATCATGATT TATTTTCTAC ATGTATTCAG 300
ACAGTACAGA AGAAAGTGTT CAAAACTCTG GATGTTTTAA TTTACAGTTA GTGGAGAAGT 360
TCGGCATTGA TCCGAACAAT GCATTTGCAT TTTGGGACTG GGTTGGTGGA AGGTACAGTG 420
GTAAGTGCTT GTTTATTTGG TTGTATAAAT TTCTCGTCCA TTTCCGCTTG CTTAGTGTAT 480
AACTGAAATT CTTTTGCAGT TTGCAGTGCT GTTGGAGTCT TACCATTGTC TCTACAGTAT 540
GGCTTCTCTG TGGTTGAGAA GTACGGTACC TTCTACTTTA TCAGCCATCT CATAAAATGT 600
CTTAGGCATA TTCTTTCTAT TTTATTTCCC TCTTAATGAT TTCTTCTTTT TTTTATTGCA 660
TTCCCGTTTT ATTTTCAAAA GTTGTTACTG TCTCTAAATC AAGAAGAAAC CTTCTTAGTA 720
GATCCAGCTG ATATTCAGCC TTTTTTAAAT TGGACTGCAG GTTTTTAAAG GGGAGCTTCA 780
AGCATTGATA AGCATTTCCA GTCCACACCG TTTGAGAAGA ATATACCCGT GAGTTGCATT 840
AGTTGTGTGA TTATACAGTT TTCTTGTCTT TTTGCTATGT CCATCAACAC TAGAGATTCG 900
TGAAGTTATT AGTGTAGTCA ACGCATAGGG AGAGGTGATT GGTGACTTTT GGACGATTTC 960
AGGTGCTTTA GGGTTATTG                                               979
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PGIint R2000 marker:

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CAGCACTAAT CTTGCGGTAT GAATTTGTGA TTAAATTTGT TTGTTTGTGA CTCTTTCTTC  60
ATTGTTCGTT TTCGTACAAT AAACCGAATG TATAATCTTT TACAAACTGA ATTTTCTACC 120
GGGTCTGATG TACAATGCTA GTCTCCATGT TCTTGGGGAT CATGATTTAT TTTCTACATG 180
TATTCAGACA GTACAGAAGA AAGTGTTCAA AACTCTGGAT GTTTTAATTT ACAGTTAGTG 240
GAGAAGTTCG GCATTGATCC GAACAATGCA TTTGCATTTT GGGACTGGGT TGGTGGAAGG 300
TACAGTGGTA AGTGCTTGTT TATTTGGTTG TATAAATTTC TCGTCCATTT CCGCTTGCTT 360
AGTGTATAAC TGAAATTCTT TTGCAGTTTG CAGTGCTGTT GGAGTCTTAC CATTGTCTCT 420
ACAGTATGGC TTCTCTGTGG TTGAGAAGTA CGGTACCTTC TACTTTATCA GCCATCTCAT 480
AAAATGTCTT AGGCATATTC TTTCTATTTT ATTTCCCTCT TAATGATTTC TTCTTTTTTT 540
TATTGCATTC CCGTTTTATT TTCAAAAGTT GTTACTGTCT CTAAATCAAG AAGAAACCTT 600
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CTTAGTAGAT CCAGCTGATA TTCAGCCTTT TTTAAATTGG ACTGCAGGTT TTTAAAGGGG 660
AGCTTCAAGC ATTGATAAGC ATTTCCAGTC CACACCGTTT GAGAAGAATA TACCCGTGAG 720
TTGCATTAGT TGTGTGATTA TACAGTTTTC TTGTCTTTTT GCTATGTCCA TCAACACTAG 780
AGATTCGTGA AGTTATTAGT GTAGTCAACG CATAGGGAGA GGTGATTGGT GACTTTTGGA 840
CGATTTCAGG TGCTTTAGGG TTATTG                                      866
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BolJon R2000 marker:

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GATCCGATTC TTCTCCTGTT GAGATCAGCT CCAAACATCA AACAACTTGT ACACAAATAT  60
CTTTACTTGC TAAATGGAAC ATGACAAGAG ATAGAAAATC TTGCTCATAG TATTGTACAA 120
GGGATAACAG TGTAGAAAAC AAACCGTCTG TAAGATTTTC TCCCTGATCC TCTCACTTAA 180
CCAGTAGGCG TTTTTCACAT TGAAGCGCAT ATCTACTTTG GTATTCACTG AATAAAAAAA 240
GAAAGCTGGT AACATGTGAA GGATATACAA GCATTGATAC ACCAAGTAGT CACAAACTAC 300
ATTATAAAGG TCAGACCTTT GTTCACATTC TGGCCTCCAG GACCACCGCT TCTAGCAAAG 360
TTAAGCGTAA CATGGTCTGC ACGTATACAA ATGAAAATGT TTCTATCAAA ATCCTATAAA 420
ATAGAGCTCT ATAACATTGT CGATACATAG TTTCACTAAC TCTGCAAGTA CTAAACACAT 480
ATACAAACAA AACTATGCGA ACAGATCAAA ACTACTACAG AACACAGTTC TATGACACTG 540
TCGATAGTAA CATCCTCTGC AAGTACCAAA GAGATAGCAA ATGAAACTAT GTAAACAAAT 600
CAAAATTCTA AATTTCTCCA TCACAAGGAC CTACAGAATA GAGTTATCAT AACATTTTCT 660
GTAAATATTT CCATCAAAAT GACTAGAGAA CAGAGTTCTT ATAACATTAT CTGTAAATGT 720
TCCAACAAAA CCACTACATA GCAGAGTTCT TATAACATTG TCTGTAAATG TCCAATCAAA 780
ACCACTACAG AACAAAGCTC CTATAACATT GTTTATACAA AGTTTCACTA AATCTACAAA 840
CTTTCCCCGT AAATGAGCTT AATATCACCC AAAGATGTTT CAATCAGATA AAGAGTACGA 900
CATCGTTTTG AGATTAGAAC AAACTGAAAC TTACGTAGAG TGATTTGAGG AGTAGGC    957
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CP418L R2000 marker:

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AATTTCTCCA TCACAAGGAC CTACAGAATA GAGTTATCAT AACATTTTCT GTAAATATTT  60
CCATCAAAAT GACTAGAGAA CAGAGTTCTT ATAACATTAT CTGTAAATGT TCCAACAAAA 120
CCACTACATA GCAGAGTTCT TATAACATTG TCTGTAAATG TCCAATCAAA ACCACTACAG 180
AACAAAGCTC CTATAACATT GTTTATACAA AGTTTCACTA AATCTACAAA CTTTCCCCGT 240
AAATGAGCTT AATATCACCC AAAGATGTTT CAATCAGATA AAGAGTAACG ACATCGTTTT 300
GAGATTAGAA CAAACTGAAA CTTACGTAGA GTGATTTGAG GAGTAGGCTC GTTGCCAGCA 360
GAGCTAGCTC TCTCCTCCGC CTCATGAAGC ATCTGTTGCA CCTGAGACAA CCGTGACGAA 420
ACTTTCCGAT CACCGCCACC AGAATTCGAC GCCGCGCATC GGAAGGATCC GAATCGGGAA 480
CTGAGTGAAC CCGAGCGATC CCGGGAGTGC GACGGAGCGA TGGGAAAAGA GAGTGGCACG 540
ATTTCGACGA AGAGTGGAAG AGGAGAGGGT GGTGGATAAA CTCGCGTATG ATCAAGTTCG 600
TCATCGTCCT GATTGCCGCC ATTTTTTTTG TCAGGGCGCT CTGTGGCTTA GAAGTTTCCG 660
ATGTCAATGA AC                                                     672
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In the annexed drawing that follows, the following abbreviations are used :

| | |
|---|---|
| Dra | Drakkar |
| Rel-15-1, E38,R15 | R2000 |
| Hete, Hel, R211.Drakkar | heterozygous R211*Drakkar, |
| Darm | Darmor |
| Bol: | Brassica oleracea |
| Bra, B.rap: | Brassica rapa |
| GCPA18-A19, Wes, Aust: | Wesroona |
| Sam, SamIPGIolSunt5 | Samourai |
| RRH1, ba2c | RRH1 |
| rav, N.WR | Hybrid Brassica napus*wild Radish |

- Figure 1 illustrates Gamma ray Iradiation and F2 production.
- Figure 2 illustrates seed set on 'R211' and 'R2000'.
- Figure 3 illustrates the number of seeds per pod of different lines.
- Figure 4 illustrates PGIol primer localisation on the segment of PGI sequence from Data Base. In that figure:

PGIol: - primer PGIol U (named in SGAP: BnPGIch 1 U)
- primer PGIol L (named in SGAP: Bn PGIch 1 L)

PGIint: - primer PGIint U
- primer PGIint L (is out side the sequence).

- Figure 5 illustrates electrophoresis gel of PGI-2 gene (PGIol), PCR marker and SG34, a PCR marker close to Rfo.
- Figure 6 illustrates Pgi-2 segment of DNA amplified by PCR with PGIol primers.
- Figure 7 illustrates digestion of the PCR product PGIol by Mse1.

In that figure:

Sam and Darm has a 75bp band.

Drak, R211.Dk and R2000 showed a 70pb one (Acrylamide 15%).

8 was similar to Samourai (75bp) ; mix with Drakkar (70pb) it allowed the visualisation of the two bands.

- Figure 8 illustrates electrophoresis agarose gel of PGIUNT marker.

In that figure:

PGIUNT band (about 980bp) is present in B.oleracea, B.rapa cv Asko, maintainer and restored lines except in 'R211'.

There is no amplification in radish and Arabidopsis.

In various Brassica genotypes only one band was amplified. Size band are similar but sequences are different.

- Figure 9 illustrates electrophoresis gel of PGIint PCR marker.

In that figure PGIint of radish line 7 is of about 950bp. This band is the same as in the restored RRH1 and R113. It is not found in R211. It is not either in R2000. However the PGIint band is of a similar size of about 870bp in the various Brassica species, but sequences are different.

- Figure 10 illustrates electrophoresis agarose gel of Bo1Jon PCR marker.
- Figure 11 illustrates electrophoresis agarose gel of CP418 marker.

In that figure, the CP418 band (of about 670bp) is specific to the B.oleracea genome. It is present in B.ol, B.napus (Samourai, Drakkar, Pactol and the herterozygous R2111*Dk). It is absent from the restored rapeseed (RRH, R113 and R211). It is present in the homozygous R2000.

- Figure 12 illustrates summary markers table.
- Figure 13 (13(a),13(b)) illustrates PGIol marker sequence alignment between Arabidopsis, Radish, B.rapa, B.oleracea and R2000.
- Figure 14 (14(a), 14(b), 14(c), 14(d)) illustrates the PGlint-UNT marker sequence alignment between Arabidopsis, Radish, B.rapa, B.oleracea and R2000.
- Figure 15 (15(a), 15(b), 15(c)) illustrates the CP418L marker sequence alignment between Arabidopsis, Radish, B.rapa, B.oleracea and R2000.
- Figure 16 (16 et 16bis) illustrates Arabidopsis, Radish and B.rapa BolJon markers. There are aligned with DB sequences of Arabidopsis (AC007190end-AC011000beginning), the B.oleracea EMBH959102 end and EMBH448336 begining and representative consensus sequences of the SG129markers band 1 and 2 in B.napus (in Drakkar and Samourai respectively).

From the point 836bp, AC07190-AC11000 and GCPATpBOJ sequences are no longer closely homologous to the Brassica sequences.

The radish and B.rapa (GCPconsen RsRf BOJ and BR) sequences are still closely homologous to the B.napus one, from 858bp point to the 900bp and 981 points respectively.

In radish, only partial homology is found on the Brassica sequence further down.

In B.rapa species cv Asko, the left of its BolJon sequence can be aligned again, after a 78bp deletion, with those of B.oleracea and B.rapa in B. napus from the 1057bp point to the BolJon L primer.

- Figure 17 (17 et 17bis) illustrates the localisation of Pgi-2 primers on the Arabidopsis th MJB21.12 sequence.
- Figure 18 illustrates the BolJon primers localisation on the mipsAtl62850 gene and overlapping area of AC007190 and AC011000 Arabidopsis th clones. Alignment with the Arabidopsis BolJon PCR product (740bp) is presented.

It should be understood, however, that the examples are given solely by way of illustration of the object of the invention, of which they in no way constitute a limitation.

**Example I: method of producing a double low restorer line of Brassica napus for Ogura cytoplasmic male sterility (cms) presenting a radish introgression, carrying the Rfo restorer gene deleted of the radish Pgi-2 allele and recombined with the Pgi-2 gene from Brassica oleracea, and having a good agronomic value characterised by female fertility, a good transmission rate of Rfo and a high vegetative vigour.**

Materials and methods :

Genotypes: The 'R211' line with a deleted radish insertion was crossed to the spring low GLS rapeseed 'Drakkar' to produce a F1 progeny ('R211*Dk'). The spring low GLS cms line 'Wesroona' (australian origin) was used for following crosses. Were used as control in molecular analyses: Winter restored lines derived from 'Samourai' carrying the complete ('RRH1') or incomplete ('R113') introgression as well as European radish line7, Asiatic restored radish D81, hybrid Brasica napus* wild radish, Brassica oleracea, and B.rapa cv Asko, Arabidopsis thaliana.

Gamma ray irradiation: Whole flowering plants were treated with gamma rays from a Co60 source in a controlled area. Subletal dose fo 65 Gray was applied before meioses.

Testcrosses and F2 production: Irradiated plants were transferred in an insectproof greenhouse after removing flower buds larger than 2 mm. The irradiated F1 progeny was used to handpollinate the cms 'Wesroona' line. The restored derived F1' plants were allowed to produce F2 families harvested individually and precisely sown in a field assay along with non irradiated controls (Fig 1).

Phenotypic selection: Three visual criteria were scored (on a 1 to 5 scale) over 2 years in field assays, on 1200 F2 offsprings plus 44 controls (82 330 quoted plants):

1-Vegetative vigour,

2- Normality of the ratio of fertile /sterile plants in the F2 segregation, and

3- Female fertility (pod development and seed set).

Advanced selfed generations of the selected families were obtained either in field or greenhouse and produced homozygous lines (F4) for further analysis.

Isozyme analysis was performed as in (Delourme R. and Eber F. 1992. *Theor Appl Genet* 85: 222-228), marker development from (Fourmann M et al 2002. *Theor Appl. Genet.* 105:1196-1206.): PCR products are validated by sequencing. Alignments were made using Blast Ncbi and Uk Crop Net Brassica DB and the Multialin software INRA Toulouse.

Method :

We choose one low GLS spring homozygous restorer line, 'R211', already exhibiting deletions in the introgression (Delourme R. and Eber F. 1992. *Theor Appl Genet* 85: 222-228. Delourme R et al 1998. *Theor Appl Genet* 97: 129-134. Delourme R. et al 1999. *10th Int. Rapeseed Congress, Canberra.)*. Several molecular markers are missing on either side of Rfo, such as spATCHIA (Fourmann M et al 2002. *Theor Appl. Genet.* 105:1196-1206), spSG91 (Giancola S et al 2003 *Theor Appl. Genet. (in press)*). 'R211' lost the isozyme expression of the Pgi-2 allele of the radish gene but also the one of Pgi-2 allele of B.oleracea genome (1,2). Moreover, the homozygous 'R211' shows linked negative traits such as low vigour and very poor seed set. We hypothesised that these plant lack a rapeseed chromosomal segment. The fertile ratio in F2 progenies derived from this material is lower than expected (64% instead of 75%). We initiated the program from this 'R211' line and tried to force recombination between the Rfo carrying introgression from this deleted line and the rapeseed homologous chromosome from a double low B. napus line.

Ionising irradiation is known to induce chromosomal rearrangements by double strand breaks followed by aberrant rejoining of the ends.Gamma-ray irradiation was used on a heterozygous F1 derived from the 'R211' line to induce chromosome breaks, just before meiosis, aiming at a recombination of the deleted radish introgression in the rapeseed genome.

Results :

Very few families were at the best score for the three criteria out of 1200 F2 families tested.

Only one, 'R2000', proved to produce a normal ratio of fertile plants per selfed progeny with a stable recovery of good agronomic traits such as a good female fertility, with a normal seed set compared to 'R211' (Fig 2 and 3). This family was obtained from a 6 mn irradiation treatment at a dose flow of 65 Gray per hour.

Glucosinolate analysis confirmed its low content.

In figure 2 (Seed set on 'R211' and 'R2000') R2000 showed normal inflorescences, with a normal looking architecture.

In figure 3 (Number of seeds per pod), we observe:

- on the best 'R2000' F4 families in self pollination (Selfings) and in testcrosses
- on 'Pactol' cms line on rapeseed and 'R211' controls.

**Example II: selection of markers in the Pgi-2 gene**

PGI isoenzyme analysis: 'R2000' progeny expressed the rapeseed Pgi-2 allele from B. oleracea genome, originally lost in 'R211'.

Three PCR markers were defined to characterise the R2000 family compared to the known restorer rapeseed RRH1 and R113.

1) PGIol marker was developed from the BrassicaDB sequences to be specific to the Brassica genome. There is no amplification in radish nor in Arabidopsis th., but only in Brassica, with one 248 bp band.

2) PGIint marker amplified a longer part of the Pgi-2 gene, allowing clear distinction between the various tested species Brassica, Raphanus and Arabidopsis. The species B.rapa and B.oleracea were not distinguished by the band size on agarose gel, but by their PGINT band sequence.

3) PGIUnt marker, a combination of the PGI ol U and PGI int L primers. This marker had the specificity of the PGIol marker but amplifying a longer part as for PGIint one.

II.1 PGIol marker

With the PGIol primers, the 'R211' parental line showed no amplification, while the spring tested lines showed a 248bp band. Its DNA sequence is homologous to the PGI-2 sequences from the Crop Net UK DB in Brassica species and from previous work in our group ( named SGAP sequences) (Localisation of the primers SG PGI chou, Fig 4).

It was ortholog of the clone MJB21-12, on the chromosome V, (34543bp) in Arabidopsis (NCBI DB).

PGIol plus SG34 to set an Homozygocity test:

The combined use of two sets of primers in a mix PCR, PGIol marking the Pgi-2 gene absent in the homozygote restored plant and SG34 (from S. Giancola et al, Giancola S et al 2003 *Theor Appl. Genet. (in press)*), a very close marker to the Rfo gene, was set up to discriminate homozygous from heterozygous plant among the fertile plants segregating in F2 progenies derived from 'R211'. In place of using SG34, it is possible to use any other marker close to or in the Rfo gene.

Only one family R2000 showed no difference between homozygote and heterozygote offsprings:

The Pgi-2 gene is present in the R2000 homozygote, which is not the case for the parental homozygous R211.

In figure 5 (PGIol and SG34 PCR markers):

The homozygous 'R2000' family has recovered the PGIol band.

DNA sequence of the band confirmed the homology with the known Arabidopsis and Brassica Pgi-2 sequence. Control genotypes (Drakkar, Pactol, and, Samourai, Darmor ) had the same pattern on the gel. Sequence of this common band allowed to confirm their high homology as they were quasi similar except one base substitution.

The homozygous 'R2000' family has recovered the PGIol band of the Brassica oleracea type. It was distinct from the known restorer of the Samourai group.

This amplified part of the Pgi-2 is very conserved and hardly any differences were shown among the various genotypes. A longer part of Pgi-2 gene was investigated.

II.2 PGIUNT and PGIint markers

Electrophoresis Patterns of PCR products:

PGIUNT marker: A second reverse primer, PGIint L, was designed further down the Pgi-2 sequence, to amplify as well conserved and as variable regions of the gene. When used with the PGIol U primer, it amplifies a 980bp band only in Brassica genomes.

R211 didn't show any band, The homozygous 'R2000' showed the PGIUNT band as in the Drakkar parent.

In figure 8 (PGIUNT marker):

PGIint marker amplified a segment of PGIUNT. The upper primer PGIint allows the amplification in all tested species, allowing a clear distinction between Arabidopsis, Radish and Brassica. B.rapa and B.oleracea were not distinguished by the band size on agarose gel, but by their PGIint sequence. All tested restored genotypes, but the 'R211' line, exhibited the European radish band and one Brassica band, homologous to the B.rapa one.

The homozygous 'R2000' didn't show the radish PGIint band, as in the deleted 'R211' parental line, but showed one Brassica band, homologous to the B. oleracea one.

Electrophoresis of PGIint marker is represented in figure 9.

Sequence analysis:

Comparison of the PGI sequences from the data bases.

A PGI segment of about 490bp is known.

Sequences of a segment of about 490bp from different genotypes (B. oleracea, B. rapa, B. napus) have been studied in our laboratory group and some sequences were given to Brassica Crop Net DB: EMAF25875 to 25788 by M.Fouramnn (4) These sequences are very conserved.

Comparison of the B. rapa et B.oleracea species PGI sequences (figures 13 and 14):

Comparison between PGI sequences we have obtained from the tested genotypes of B.oleracea and B.rapa species, showed that they were distinct by 21 base substitutions. Theses substitutions allowed to distinguish PGIint sequences from the other tested genotypes of rapeseed, homologous to either B.rapa cv Asko (RRH1 and R113) or B.oleracea (Drakkar, R211*DK but also R2000).

**Example III: selection of marker in a region close to Rfo**

Markers surrounding the Rfo gene in the radish insertion were determined in order to facilitate the Rfo gene cloning (Desloires S et al 2003 *EMBO reports* 4, 6:588-594). One of these, the SG129 PCR marker was located very close to Rfo (Giancola S et al 2003 *Theor Appl. Genet. (in press)*): it co-amplified distinct bands in B.oleracea and B.rapa genomes of B.napus, but the radish band was very difficult to see on an agarose gel.

The target SG129 sequence was ortholog of a clone (AC011OOO, at the locus F16P17) in Arabidopsis thaliana. This clone overlapped an Arabidopsis adjacent contig clone (AC07190).

From the Brassica Crop Net DB, we found one B.oleracea clone, (EMBH448336, 764bp) blasting with the begining of the A011000, and a second B.oleracea clone (EMBH53971), distant from about 300bp on the Arabidopsis map, that blasted with the end of ACO7190.

We designed a new PCR marker, BolJon, between the two B.oleracea clones. We verified that it allowed amplification of a specific PCR bands in the different genotypes compared here.

In figure 16 (electrophoresis gel of BolJon PCR products):

- In Arabidopsis, a BolJon 815bp band was amplified, homologue to the overlapping segment of the contigs.
- In Brassiceae diploid species, BolJon marker showed distinct bands: one of 950bp in B.oleracea and one of 870bp in B.rapa. It showed that the two B.oleracea clones (EMBH53971 and EMBH448336) are in sequence continuity in Brassica genome as it is for the ortholog sequences in Arabidopsis.
- In B.napus, these two bands are co-amplified in the maintainer lines, Samourai or Drakkar.
- In radish line7, one BolJon band was amplified of about 630 bp long. The band of the restored radish cmsRd81 was slightly smaller.
- In all the restored rapeseed lines, one of the BolJon bands was of the same size as the radish line7. BolJon is a marker of the radish introgression.
- The homozygous restored rapeseed lines, 'RRH1', 'R113' and also 'R211', only showed the B.rapa band and the 630bp radish band bp suggesting the B.oleracea ortholog of the target gene is absent or has been modified when the radish segment of chromosome was inserted into the rapeseed B.oleracea constitutive genome.

'R2000' homozygote plants showed radish PCR BolJon, plus the two Brassica BolJon bands, again having recovered the B.oleracea one, lost in 'R211' and other restorer lines.

We designed a primer, pCP418L, specific of the B.oleracea genome in the tested species. With the SG129U primer it amplified only one PCR band (670bp) in the B.oleracea species. (Fig 17)

There was no amplification in B.rapa, in radish, nor in Arabidopsis, but there was a clear CP418 band in B. napus maintainer lines. Its sequence was strictly homologous to the EMBH448336 sequence. This marker was in a very conserved DNA sequence allowing no polymorphism between genotypes except by presence / absence.

In RRH1, R113 and in R211 there was no CP418 band, indicating as previously that the B.oleracea ortholog of the target gene is absent or has been modified following the radish insertion.

'R2000' homozygote plants showed CP418 band, again having recovered the specific B.oleracea one.

In the present invention, a new recombined low GLS restorer line has been selected with a good female fertility. The poor value of line 'R211' allowed selection in the field for a rare recombination event and characterisation the 'R2000' family.

The homozygous 'R2000' presents a unique combination of the PGIol, PGIUNT, PGIint and BolJon markers when compared with the rapeseed restorer analysed yet:

PGIinT marker showed that the homozygous restored rapeseed lines, RRH1 and R113 presented the European radish band plus one Brassica band, homologous to B.rapa genome. 'R2000' shows no radish band, lost as in its parental deleted line R211, but showed one Brassica band homologous to B.oleracea. The ortholog PGIint sequence in its B.rapa genome is not amplified with this marker in R211 and Drakkar genetic background.

PGIol marker and PGIUNT marker sequences in restored lines RRH1 and R113 were homologous to the B.rapa cv Asko one. In 'R2000', PGIUNT sequence is homologous to B.oleracea. The ortholog PGIUnt sequence in its B.rapa genome is not amplified with this marker in R211 and Drakkar genetic background.

BolJon marker showed that the homozygous restored rapeseed lines, including 'R211' presented the European radish band plus only the B.rapa one. 'R2000' shows the two bands of 'R211' plus the recovered B.oleracea BolJon band.

CP418 marker showed that 'R2000' recovered this conserved B.oleracea segment.

Our hypothesis is that a recombination event took place in the pollen mother cell which gave rise to 'R2000' plants. The deleted radish introgression was then integrated to the normal homologous chromosome segment, carrying the B.oleracea type Pgi-2 gene and BolJon target sequence, characterised by these markers, probably from the Drakkar '00' genome present in the irradiated heterozygous 'R211*DK'.

The pattern observed for BolJon suggests that the recombination event resulted in a particular duplicated region, one from radish and one B.oleracea, in the 'R2000' family.